



## Biological effects of agricultural bio-materials on some blood and tissue factors in Balb/c mice

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### ABSTRACT

*Pseudomonas* infections are an important cause of morbidity and mortality and saprophytic fungi are now increasingly being recognized as serious pathogens in immunocompromised patients. To investigate the effect of using bio-materials on mammalian tissues, two experiments were designed; the first one was feeding of Balb/c mice with irrigated lettuce with bio-fungicide (mutant and wild) and bio-fertilizers prepared with *Pseudomonas* (*p. fluorescens*, *p. putida*, *p. aeruginosa*), and the second was the usage of drinking water containing (*Trichoderma* (T) spores (mutant and wild) or *P. fluorescens*, *P. putida*, *P. aeruginosa* suspensions). Then, blood factors and inflammation of tissues (liver, kidney, spleen and large intestine) in all mice were analyzed after two months. Blood samples were taken from the mice to examine some of the hematological (RBC, MCV, MCH, MCHC) (data not shown) and biochemical (AST, ALT, ALP) factors, and also observed under a microscope. The study of tumor marker carcinoembryonic antigen (CEA) in all treatments showed that the strains in these bio-fertilizers did not stimulate carcinogenic indices. The results from the other blood factors were normal for all strains (data not shown). Only *P. putida* showed no adverse effect on the increase in alkaline phosphatase (ALP). The results also showed that the effect of bio-fungicide on mammalian tissues (spleen and large intestine) was normal. But a small number of mild liver necrosis was seen in the treatment groups with wild *Trichoderma*, and moderate necrosis in the liver tissue after treatment with mutant *Trichoderma* isolates. More investigation should be made to determine the impact of these biotic factors on the mammalian tissues before commercialization.

### Keywords

*Pseudomonas*, Fertilizer, Biological Fungicide, *Trichoderma*

### Abbreviations

CEA: Carcinoembryonic antigen  
ALP: Alkaline phosphatase  
P: *Pseudomonas*  
T: *Trichoderma*

Introduction

According to the statistics published in Iran, per capita consumption of poison in agricultural products for each person is 400 grams, and the use of chemical fertilizers has been increased from 2.5 to 3.5 million tons over the past 10 years. In the traditional agriculture, over 300 types of hazardous chemicals such as pesticides, herbicides, and fertilizers are used to control pests, insects and to increase soil fertility. The residue of these materials will be absorbed into plants by infecting groundwater and air. In addition, it will accumulate in agricultural products such as fruits and vegetables and will be transmitted to the human body. For many years, to tackle these problems, the bio-fungicides such as commercial products made by *Trichoderma* and bio-fertilizers made with *Pseudomonas* have been used in different countries (1). *Pseudomonas* spp. is an aerobic, gram negative, rod shaped, non-spore forming and fast growing bacterium. The most important fluorescent species are *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Pseudomonas fluorescens* (2). *Pseudomonas fluorescens* unlike *P. aeruginosa* has generally been regarded to be of low virulence, and an infrequent cause of human infection (3). However, it has been reported to cause infections such as blood transfusion-related septicemia (4, 5), catheter-related bacteremia (3), and peritonitis in peritoneal dialysis patients (6). *Pseudomonas aeruginosa* being the most common species isolated from clinical specimens (7). Its pathogenicity has generally been related to its exotoxin. These exotoxins can produce leukopenia, acidosis, circulatory collapse, necrosis of liver, pulmonary edema, hemorrhage, and tubular necrosis of kidneys. The extracellular toxins that cause damage to the tissues of different hosts may differ. *Pseudomonas aeruginosa* causes several different infections including endocarditis, pneumonia, malignant otitis externa, bacteremia, and also, gastrointestinal tract, skin and soft tissue, skeletal, eye, and burn infections (8, 9). *Pseudomonas putida* is an uncommon cause of skin and soft tissue infections. It is often associated with trauma or immunocompromised states, and in patients possessing medical devices or catheters (10, 11). *Pseudomonas putida* is considered a low-virulence pathogen and has been recognized as a rare cause of bacteremia. Despite the fact that this organism causes health care-related infections, clinical data on *P. putida* infections are lacking, owing to the rarity, relatively lower virulence, and higher antimicrobial susceptibility of *P. putida* compared with other *Pseudomonas* species, especially *Pseudomonas aeruginosa* (10, 12-14).

It is necessary to reduce the consumption of chemical fertilizers and pesticides on greens and veg-

etables, such as lettuce, which are eaten fresh. However, problems with residual pesticides and pathogenic chemical compounds indicate that the production and use of bio-fertilizers has become more important. The current research was conducted to evaluate the possibility of undesirable effects from these organisms in greenhouse cultivation systems, groundwater and the mammalian food chain.

*Trichoderma* spp., has been widely investigated in recent years and is the most widely used as a bio-control agent against phytopathogens (15). Over the past decade, infections caused by opportunistic filamentous fungi have become increasingly common among patients after allogeneic stem cell transplantation (allo- HSCT). *Trichoderma* species are considered plant saprophytes, but have recently been linked to severe cases of invasive infection in immunocompromised human hosts [16-19]. The aim of this study was to investigate the biological effects of *Trichoderma rifaii* (mutant and wild types) on Balb /c mice (as a model mammal). Obviously, a more comprehensive study should also be carried out on the other isolates that are used as bio-fungicides and pesticides in the production systems. The goal is to draw the attention of other researchers, before the recommendation and application of bacterial microorganisms. The possible adverse effects of inoculation with different species of *Pseudomonas* spp. and the risks associated with the use of these biological compounds on mammalian health has also been investigated.

Results

The present study examined the biological effects of these bacteria and *Trichoderma* as a bio fungicide on inflammation or necrosis of tissues such as kidney, liver, spleen and large intestine, blood factors (alkaline phosphatase), carcinoembryonic antigen (CEA) and hemoglobin in a mammalian model (mouse). The study of tumor marker CEA in all treatments showed that the strains in these bio-fertilizers did not stimulate carcinogenic indices. The results from the blood factors were normal for all strains (data not shown). Only *P. putida* showed no adverse effect on the increase in alkaline phosphatase (ALP). In this study of the inflammatory processes in the liver, kidney and large intestine, of the 12 mice studied, only minor liver and kidney necrosis and the large intestine necrosis were observed for the different bacterial strains (Figure 1). No pathologies were found in the spleen tissue (Figure 1). The data acquired from 12 mice indicates the need for further studies on the effects of bio-fertilizers on mammals (Table 1). The results of blood factors ALP and CEA in all treatment groups showed

that the bio-fungicide propagule did not stimulate carcinogenicity indices. The study of inflammatory process in the liver and kidney of the twelve studied mouse tissues showed that a small number of mild liver necrosis were seen in the treatment with wild-type *Trichoderma*, and moderate necrosis in the liver tissue after treatment with *Trichoderma* mutant isolates (Figure 1-b2). There were no effects on the spleen and large intestine (Table 2) (Figure 1c, d2).

Discussion

According to the results of this study, tumor marker CEA was not affected by different bacterial strains. Similar results were reported by Kiyama et al. (20). Blood factors were normal in all groups treated with all strains (data not shown). Only *P. putida* showed no adverse effect on the increase in ALP (21, 22).

The pathological changes in the liver, spleen, lung and heart were similar to the changes reported by Al-Muhammadawi (23). Olgerts et al., (24) reported the histopathology and serum enzyme levels of mice inoculated intravenously with *Pseudomonas*

*aeruginosa* exotoxin. The toxin had a significant effect on the liver but did not cause any microscopic changes in other organs. Microscopic changes resulting from an injection of two 50% lethal doses (LD50) of toxins (2.3 g) into the liver are characterized by necrosis, cell swelling, and fat change within 4-8 hours and it was similar to necrosis of cells in the kidney, after 48 hours. Liver necrosis was associated with a parallel increase in serum levels of aspartate and alanine aminotransferases and alkaline phosphatase.

A single injection of 10 LD50 elicited similar but somewhat more rapid degeneration. No progressive lesions were seen after injection of toxoid or of 0.5 LD50 of toxin. Our results were similar to these results. Our microscopic observations are similar with those made by Liu (25). He has briefly reported liver necrosis, edematous and hemorrhagic lungs, and necrotic and hemorrhagic kidneys in mice given intraperitoneal toxin. The enzyme activity in serum (a relatively small increase in levels of alkaline phosphatase) was consistent with the histologic pattern of necrosis.

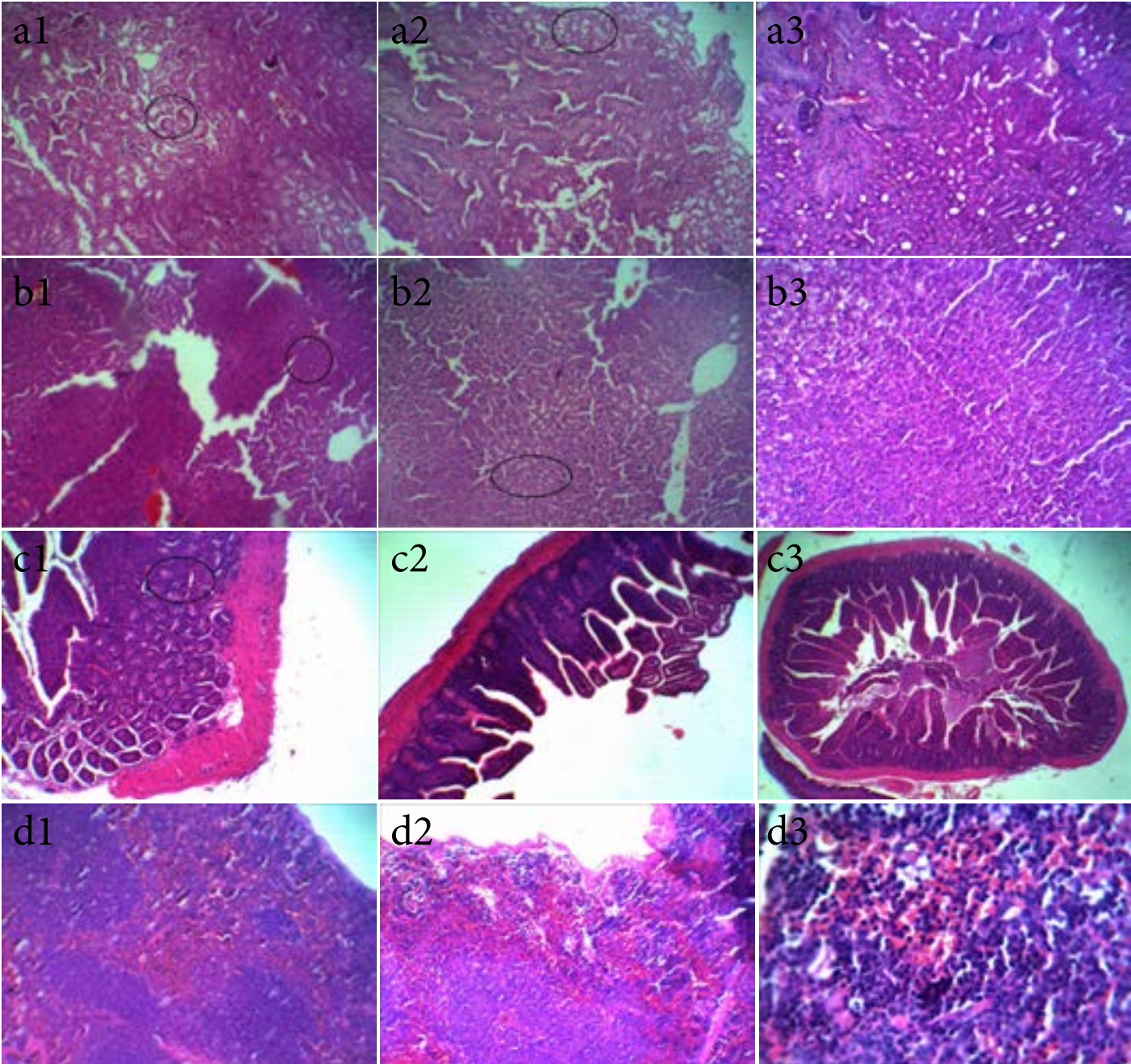
Based on the Rees (26) report, *Trichoderma harzianum* strain T-39 was not infectious, pathogenic or toxic to rats when administered orally at 1.4 to 2.0 x

**Table 1**  
Effect of bio-fertilizer on blood factors and inflammatory processes in extremities of mice treated with bacterial cells and lettuce.

| Treatment                         | Blood                                    |                                         | Vital organs |        |       |                        |
|-----------------------------------|------------------------------------------|-----------------------------------------|--------------|--------|-------|------------------------|
|                                   | CEA <sup>2</sup><br>Normal (<2.5 ng/ml ) | ALP <sup>1</sup><br>Normal (230-55 U/L) | Kidney       | Spleen | liver | gastrointestinal tract |
| Control (tap water)               | 0.31                                     | 139                                     | 0            | 0      | 0     | 0                      |
| <i>P. fluorescens</i> + (water)   | 0.39                                     | 485                                     | 0            | 0      | 1     | 1                      |
| <i>P. putida</i> + (water)        | 0.21                                     | 142                                     | 1            | 0      | 0     | 0                      |
| <i>P. aeruginosa</i> + (water)    | 0.3                                      | 296                                     | 1            | 0      | 1     | 1                      |
| Control (lettuce)                 | 0.27                                     | 129                                     | 0            | 0      | 0     | 0                      |
| <i>P. fluorescens</i> + (lettuce) | 0.33                                     | 330                                     | 0            | 0      | 0     | 1                      |
| <i>P. putida</i> + (lettuce)      | 0.29                                     | 165                                     | 1            | 0      | 1     | 0                      |
| <i>P. aeruginosa</i> + (lettuce)  | 0.25                                     | 288                                     | 0            | 0      | 1     | 0                      |

<sup>1</sup> alkaline phosphatase  
<sup>2</sup> carcinoembryonic antigen  
Ranked inflammatory process: negative=0; mild necrosis=1; moderate necrosis=2; severe necrosis=3





**Figure 1.** Comparison of microscopic image of different organ tissues treated with biological materials. a1: The kidney tissue of mouse fed with *P. putida*. a2: The kidney tissue of mouse fed with mutant *Trichoderma*. a3: The kidney tissue of control. b1: The liver tissue of mouse fed by *P. aeruginosa*, b2: The liver tissue of mouse fed with mutant *Trichoderma*. b3: The liver tissue of control. c1: The gastrointestinal tract tissue of mouse fed with *P. fluorescens*, c2: The gastrointestinal tract tissue of mouse fed with mutant *Trichoderma*. c3: The gastrointestinal tract tissue of control. d1: The spleen tissue of mouse fed with *P. putida*. d2: The spleen tissue of mouse fed with mutant *Trichoderma*. d3: The spleen tissue of control. Dark ovals in the shapes indicate the presence of necrosis or cell degeneration. The nucleus becomes swollen and dark and eventually disappears

10<sup>8</sup> CFU/animal. Clearance and infectivity were evaluated in the brain, blood, lymph nodes, kidney, liver, spleen, lungs, caecum and feces. According to Leuschner's (27) findings. Rats were given an oral dose of *trichoderma asperellum* strain ICC 012 (*Trichoderma asperellum conidia* 4.2 x 10<sup>9</sup> CFU/g) at 6-7 weeks of age, then the mice were evaluated over a period of 14 days. The results of this study showed that the *Trichoderma* strain was not toxic at a concentration of 2000 mg / kg body weight. No deaths occurred during the study. *Trichoderma* infection and pathogenesis are also unknown and no clinical signs of treatment or weight change were observed.

According to a report, a suspected case of invasive pulmonary infection with *T. longibrachiatum* in a patient with severe aplastic anemia who received allo-HSCT and was successfully treated with liposomal amphotericin B (L-AmB). There are few reports on the effects of *T. harzianum* on mammalian cells (28) or humans, while the effects of biologically active peptides produced by other *Trichoderma* species have been extensively investigated (29).

According to the Biotechnology Committee of biological products the total cultivated area of crops produced in Iran without the use of pesticides and fertilizers is about 239 thousand and 364 hectares, including 125 thousand and 802 hectares of horticultur-

al products and 113 thousand and 659 hectares of agricultural crops. Generally, the amount of cultivation of agricultural and horticultural that produce them without using fertilizers and poisons is 1% and 2.7% of the total cultivated in Iran, respectively.

In order to establish a bio-agronomic system in Iran, at the same time as the development of this production, the reliable and verified planning in the field of supply these compounds in the distribution network is essential because economization of bio-agriculture is necessary for its development and expansion. Before all this, accurate and sequential reviews on the effect of the use of this fungicide and bio-fertilizers on consumers in long-term should always be included in the agricultural research program in order to prevent the potential harm of such compounds or to make informed choices based on scientific data to the consumer.

**Conclusion**

The results for the effect of bio-fertilizers on mammals show that they have a minor effect on the liver, kidney and large intestine. The effect of active bio-fertilizers on blood factor such as Carcinoembryonic antigen (CEA) and Alkaline phosphatase(ALP) were normal for all strains. The results also show that the effect of bio-fungicide on mammal's tissues (spleen

**Table 2**  
Effect of active fungicide on blood factors and inflammatory process in vital organs of mice treated with suspension of *Trichoderma* wild type and mutant spores and lettuce treated with fungicide.

| Treatment                              | Blood                                       |                                            | Vital organs |        |       |                        |
|----------------------------------------|---------------------------------------------|--------------------------------------------|--------------|--------|-------|------------------------|
|                                        | CEA <sup>2</sup><br>Normal<br>(<2.5 ng/ml ) | ALP <sup>1</sup><br>Normal<br>(230-55 U/L) | Kidney       | Spleen | liver | gastrointestinal tract |
| Control (water)                        | 0.31                                        | 139                                        | 0            | 0      | 0     | 0                      |
| Wild type <i>Trichoderma</i> + water   | 0.32                                        | 170                                        | 0            | 0      | 1     | 0                      |
| Mutant <i>Trichoderma</i> + water      | 0.33                                        | 290                                        | 1            | 0      | 1     | 0                      |
| Wild type <i>Trichoderma</i> + lettuce | 0.10                                        | 250                                        | 0            | 0      | 1     | 0                      |
| Mutant <i>Trichoderma</i> + lettuce    | 0.42                                        | 198                                        | 1            | 0      | 2     | 0                      |
| Control (lettuce)                      | 0.27                                        | 129                                        | 0            | 0      | 0     | 0                      |

<sup>1</sup> alkaline phosphatase  
<sup>2</sup> carcinoembryonic antigen  
Ranked inflammatory process: negative=0; mild necrosis=1; moderate necrosis=2; severe necrosis=3



and large intestine) was normal. But a small number of mild liver necrosis were seen in the treatment with Trichoderma, and moderate necrosis in the liver tissue after treatment with mutant Trichoderma isolates that it reminds to researchers that the observation of some mild liver necrosis requires a re-examination and double-check on the effects of bio-fungicides.

The protocol of the research entitled Biological effects of Agricultural bio-materials on some blood and tissue factors in Balb/c mice was performed according to Iranian animal ethics society and local university rules.

## Material and methods

### Bio-materials

Three commercial bio-fertilizers containing *P. aeruginosa*, *P. putida* and *P. fluorescens* were obtained from the Soil and Water Research Institute in Karaj, Iran. *T. rufaii* species (wild and mutant isolates) were collected from fungal collections of the Karaj Nuclear Agriculture Research Center. The tested mice were white, male and female, prepared from the Razi Vaccine and Serology Research Institute.

### The effect of *Trichoderma* spp and *Pseudomonas* on mammals

The bio-fungicide propagule (suspension of the spore from isolated wild and mutant *Trichoderma*, with a concentration of  $10^7$  cells per ml) and the bio-fertilizer propagule (suspension of bacterial cells with a concentration of  $10^5$  cells per ml) were added to drinking water for two months, two times per week to the first group of Balb/c mice (3 males and 9 females). In the second group, mice (3 males and 9 females) were fed with lettuce treated with bio-fungicide and three biological fertilizers three times per week. Blood factors (ALP, AST, CBC), tumor marker carcinoembryonic antigen (CEA), hemoglobin and the inflammatory process of tissues such as kidney, liver, spleen and large intestine in all mice were examined. Only alkaline phosphatase (ALP) and CEA as the main carcinogens are presented here (Tables 1, 2).

### Preparation of tissues and blood samples

Mice were 3.5-4 months old and of balb/c strain. The average weight of balb/c mice was from 20 to 25 grams. At the end of the mentioned time (50 days), the mice were anesthetized by peritoneal injection, and after the completion of the procedure, organs such as the spleen, kidney, liver and large intestine were sampled. The tissue samples were fixed in 10% formalin. After the fixation, the digestion and molding stages were done by alcohol and paraffin, respectively. Then, the transverse sections of the anterior, middle, and posterior tissues were prepared by a microtome. The slides were stained with hematoxylin and eosin and then observed by an optical microscope. Blood samples were directly taken from the heart by an insulin syringe, so that the needle was inserted into the area by observing the heartbeat, and when the heartbeat was felt as vibration of the syringe, complete blood sampling was done. After blood sampling, the samples were transferred to two vials with and without anti-coagulant EDTA. The EDTA-free samples were used for analysis of liver enzymes.

### Statistical analysis

Data obtained from the experiments was analyzed in SPSS

(ver. 13). The groups were compared using ANOVA followed by Duncan's multiple range test at the ( $p < 0.05$ ) level of significance.

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## Author Contributions

Conceived and designed the experiments: S.S, H.D. Performed the experiments: S.S. Analyzed the data: S.S. Research space and equipment: R.M, H.D, S.S. Contributed reagents/materials/analysis tools: R.M, H.D, S.S. Wrote the paper: R.M, S.S. Read and edit the paper: S.B, P.A.R..

## Conflict of Interest

The authors declare that they have no competing interests.

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